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Robust conversion of marrow cells to skeletal muscle with formation of marrow-derived muscle cell colonies: A multifactorial process

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Objective. **Murine marrow cells are capable of repopulating skeletal muscle fibers. A point of concern has been the "robustness" of such conversions. We have investigated the impact of type of cell delivery, muscle injury, nature of delivered cell, and stem cell mobilizations on marrow-to-muscle conversion.**

Methods. **We transplanted green fluorescence protein (GFP)-transgenic marrow into irradiated C57BL/6 mice and then injured anterior tibialis muscle by cardiotoxin. One month after injury, sections were analyzed by standard and deconvolutional microscopy for expression of muscle and hematopoietic markers.**

Results. **Irradiation was essential to conversion, although whether by injury or induction of chimerism is not clear. Cardiotoxin- and, to a lesser extent, PBS-injected muscles showed significant number of GFP^+ muscle fibers, while uninjected muscles showed only rare GFP^+ cells. Marrow conversion to muscle was increased by two cycles of G-CSF mobilization and to a lesser extent by G-CSF and steel or GM-CSF. Transplantation of female GFP to male C57BL/6 and GFP to ROSA26 mice showed fusion of donor cells to recipient muscle. High numbers of donor-derived muscle colonies and up to 12% GFP^+ muscle cells were seen after mobilization or direct injection. These levels of donor muscle chimerism approach levels that could be clinically significant in developing strategies for the treatment of muscular dystrophies.**

Conclusion. **In summary, the conversion of marrow to skeletal muscle cells is based on cell fusion and is critically dependent on injury. This conversion is also numerically significant and increases with mobilization.** © **2004 International Society for Experimental Hematology. Published by Elsevier Inc.**

Stem cell systems have traditionally been felt to be hierarchical in nature, characterized by differentiative potential restricted to the lineages represented by its tissue of origin. Recent studies have changed this picture dramatically. Marrow stem cells have been shown to be capable of giving rise to a large number of non–marrow cell types. Studies have shown that marrow cells can give rise to bone [1], cartilage [2], endothelial [3,4], lung [5], hepatic [6,7], neuronal [8], cardiac [9], and skin cells [10], to cite a noninclusive list.

Wakitani et al. [11] were the first to show that mesenchymal stem cells from rat bone marrow have the ability to differentiate into myotubes when they are cultured in the presence of 5-azacytidine. In vivo transplantation of such cells into the muscles of X-chromosome-linked muscular dystrophy (mdx) mice induces dystrophin-positive muscle fibers [12]. Other investigators have reported that transplanted bone marrow cells participate in the muscle regeneration process in irradiated recipient mice [13,14]. In these studies, bone marrow cells were distinguished from recipient cells by donor-specific gene expression signals such as nuclear ß-galactosidase or the presence of a Y-chromosome. However, muscle fibers containing donor cell–derived chromosomes never exceeded 1% of the total fibers in nondystrophic mice. Fukada et al. [15] demonstrated up to 2% green fluorescence protein (GFP)-positive muscle fibers after cardiotoxin injury in a model of marrow transplants from GFP-transgenic to C57BL/6 mice. In dystrophic mice a higher number of conversion has been observed. Gussoni et al. [16], for example, showed up to 4% marrow-to-muscle conversion in *mdx* mice 12 weeks after transplant. Recently, LaBarge et al. [17] used GFP-transgenic mice as a source

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for marrow cells and anti-GFP antibody as their cell tracing marker and showed that in a stress-induced muscle injury, transition of bone marrow cells to muscle satellite cell to muscle fiber results in a contribution to as many as 3.5% of muscle fibers in a muscular dystrophy model.

There has been a significant amount of controversy surrounding the issue of stem cell plasticity. Questions of the cell identification, "robustness" of the phenomenon, and whether fusion is a predominant mechanism have been highlighted. In this report we demonstrate that the intravenous or direct intramuscular injection of bone marrow cells from EGFP-transgenic mice into GFP-chimeric C57BL/6 mice establishes bone marrow–derived muscle cells. As determined by the presence of GFP and colocalization of lineage markers and by using both standard and deconvolutional fluorescent microscopy, we have established the importance of injury and mobilization, showing up to 12% donor-derived muscle fibers. We are also reporting the presence of donor-derived muscle colonies.

Material and methods

Experimental animals

C57BL/6 (H2Kb) mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were certified to be pathogen free, housed in our animal facility, and given ad libitum access to food and acid water. GFP-transgenic breeding pairs C57BL/6-TgN(ACTbEGFP)1Osb and ROSA26 breeding pair were purchased from The Jackson Laboratory. Animals were bred in our animal facility by mating heterozygous GFP^+ animals to C57BL/6 for GFP-transgenic animals and homozygote to homozygote in the case of ROSA26. Heterozygote GFP animals were separated by using a UV light source.

Bone marrow transplantation

Six- to 8-week-old mice were used as donors or recipients. After sacrificing the mice and dissecting the femur, tibia, and pelvic bones, bone marrow was obtained by flushing the bones with Hanks' balanced salt solution (HBSS) with 2% heat-inactivated fetal calf serum (HI-FCS) using a syringe and a 22-gauge needle. After resuspension in HBSS without FCS with a 5-mL pipette, cells were passed through a 40-μm cell strainer. Cell numbers were counted in crystal violet and viability was assessed by trypan blue staining. Approximately 4 hours after radiation, unselected bone marrow cells were injected intravenously by tail vein into each recipient. In most of the experiments 20 to 25 million marrow cells were infused at the time of transplantation, with the exception of mobilization experiments, where 40 million GFP^+ marrow cells were given, and experiments without radiation, where very high dose (up to 100 million) marrow cells were infused to enhance the engraftment. In a few of the experiments, in addition to the intravenous marrow infusion, GFP^+ whole bone marrow or a lineage-negative subpopulation of marrow cells, suspended in 100 μL of phosphate-buffered saline (PBS), were directly injected into anterior tibialis muscle with a 26-gauge needle. Direct injection was performed the next day after injury with cardiotoxin. A photon-producing linear accelerator (Elekta) was used for radiation of animals 4 hours before each transplant. Radiation was given at a dose rate around 85 cGy per minute. Radiation in earlier experiments was given at 900 cGy of whole-body irradiation. We later found that 500 cGy of whole-body and 500 cGy of lower-extremity radiation were better tolerated and gave quantitatively similar or superior results, so later experiments including direct injection of marrow into muscle or experiments on cell fusion were done with this split-dose radiation.

Muscle injury

We evaluated the dose of cardiotoxin (Naja mossambica mossambica, Sigma, St. Louis, MO, USA) appropriate to induce a visible lysis of muscle fibers in 48 hours. H&E staining of frozen sections of injected muscle showed that 10 mM cardiotoxin in 100 μL of PBS will induce 80 to 90% lysis of muscle fibers in the area of injection. The left tibialis anterior muscle, either uninjected or injected with 100 μL of PBS, was used as control for each experiment. Cardiotoxin diluted in 100 μL of PBS was injected into the anterior tibialis muscle using a 27-gauge needle and a 1-mL syringe. Animals were first anesthetized by halothane. The needle was inserted deep into the anterior tibial muscle longitudinally towards the knee from the ankle. Cardiotoxin was injected along the length of the muscle. Left tibialis anterior muscle was used as control.

Mobilization protocols

Three different types of mobilization were used:

1) G-CSF 250 μg/kg once a day for 5 days. In some of the groups this was repeated for a second cycle one week apart.
2) GM-CSF (R&D Systems, Minneapolis, MN, USA) 1 μg/mouse per day for 5 days.
3) SCF 200 mg/kg/day plus G-CSF 50 μg/kg for 5 days.

All injections were subcutaneous using 28-gauge Tuberculin syringes. The final volume of each injection was 100 μL.

Immunofluorescence staining

To evaluate cells included in the regenerating muscle, specimens were collected after sacrificing mice by cervical dislocation after halothane anesthesia. Excised muscle specimens were placed in freshly prepared PLP fixative solution (balanced phosphate solution with 2% paraformaldehyde, sodium m-periodate, and L-lysine) for 2 hours at 4°C, with frequent agitation. Samples were then washed in 7% sucrose buffer at 4°C overnight followed by 15% sucrose buffer for 2 to 3 hours and then 25% sucrose plus 10% glycerol buffer at 4°C for another 2 hours. They were then rinsed in HBSS and embeded in OCT, frozen, and stored at −70°C until sectioning. Immunofluorescence staining was performed on 10- to 16-micron cryosections. In the case of intracellular antigens, permeabilization was performed with 0.3% Triton-X-100 for 20 minutes. Sections were then blocked for 30 minutes with normal serum. In the case of anti-dystrophin antibody, MOM kit (Vector) was used for blocking. Sections were then incubated with rabbit anti-desmin and mouse anti-dystrophin, biotinylated anti-mouse CD45, or Alexa Fluor 488 conjugated anti-GFP antibodies (Molecular Probes, Eugene, OR, USA) for 2 hours at room temperature, followed by 1 hour incubation with respective secondaries (Alexa Fluor 594 anti-rabbit for desmin, rhodamine anti-mouse for dystrophin, and Alexa Fluor 594 streptavidin for CD45). Either GFP expression or antigens stained by different fluorescence-conjugated antibodies were visualized by fluorescent microscopy Axiovert w 135 (Carl Zeiss, Oberko-chen, Germany).

X-gal staining

Frozen sections were rinsed in PBS, fixed in PLP containing 2% formaldehyde, rinsed in detergent buffer (PBS, 0.02% NP40, 0.01% deoxycholate, 2 mmol/L $MgCl_2$), and stained at 37°C in the dark overnight. Staining solution contained 1 mg/mL X-gal [5-bromo-4-chloro-3-indolyl—D-galacto-pyranoside], 5 mmol/L K3Fe(CN)6, 5 mmol/L K4Fe(CN)6, and 1 mmol/L EGTA (ethylene glycol-bis(-aminoethyl ether) N, N, N′, N′-tetraacetic acid) in detergent buffer.

Combined immunofluorescence and FISH

Muscle specimens were sectioned at 10-micron sections baked at 37°C for 30 minutes. They were permeabilized with 1% Triton-X-100 for 20 minutes and were blocked for 20 minutes at room temperature with goat serum. They were then incubated with the anti-GFP antibody for 2 hours and fixed again with 2% paraformaldehyde and hybridized with a digoxigenin-labeled murine Y-chromosome painting probe at 45°C overnight. Unbound probe is removed by stringent washing in 4 changes of 0.8× SSC at 45°C. The slides were blocked using a blocking buffer consisting of 5% fetal bovine serum (FBS), 5% nonfat milk (Shaw's, Bridgewater, MA, USA), and 0.05% Triton X-100 (Sigma) in 4× SSC for 20 minutes. Detection of digoxigenin was done using anti-digoxigenin-rhodamine, Fab fragments (Boehringer Mannheim, Indianapolis, IN, USA), incubated in the dark for one hour. Nonbound antibody was removed through 3 light-protected washings, each for 5 minutes at room temperature [1]. Finally, slides were mounted in the anti-fade media vectashield (Vector, Newcastle, UK) with 0.4 µmol DAPI (4,6-Diamidino-2-phenyindole) (Sigma, St. Louis, MO, USA), which counterstains the DNA, and examined using the Zeiss Axioplan 2 microscope. The software platform used for these studies is AxioVision. AxioVision has a modular image processing and analysis system for use in modern microscopy. One of these modules, Z-Stack, allows the computer and the motorized microscope to take images on a Z plane. Using the Multichannel 3D Deconvolution module makes it possible to improve the quality of Z-stack images using deconvolution algorithms and the generation, automation, and enhancement of 3D images.

Flow cytometry

Blood chimerism was determined by obtaining peripheral blood from each mouse by tail vein bleeding. Fifty microliters of blood was incubated for 10 minutes at room temperature with 1.3 mL ice-cold erythrocyte lysing solution (150 mM NH_4Cl, 10 mM $NaHCO_3$, 1 mM EDTA, pH 7.4), washed with PBS and resuspended in PBS and 1% paraformaldehyde (Sigma, St Louis, MO, USA) and kept at 4°C until analysis. Peripheral donor chimerism was assessed by FACS. The percent of GFP^+ cells was calculated by the total number of GFP^+ over total number of negative cells after subtracting the background.

Lineage depletion

Bone marrow was isolated from the iliac bones, femurs, and tibiae of GFP-transgenic mice of 6 to 8 weeks of age. Cells were passed through a 25-gauge needle for making a single-cell suspension. The cells were then lineage depleted by adding the following antibodies: antiTer119, B220, Mac-1, GR-1, CD4, and CD8. After 15 minutes of incubation, Dynabead M450 anti-rat IgG (Dynal; Lake Success, NY, USA) was added and lineage-positive cells were removed by a magnetic column. Details of lineage depletion were published before [18].

Counting and statistics

To count the number of GFP^+ muscle fibers, at least 8 sections at 16 microns, each 100 micron apart, were made in each muscle specimen and the number of GFP^+ muscle fibers was counted. We also counted three random high-power fields (HPF) in each section and counted the total number of HPF for each section and then calculated a ratio between GFP^+ muscle fibers and total estimated muscle fibers (i.e., average of cell number in HPF multiplied by the number of HPF per section). We used the nonparametric Wilcoxon rank-sum test for comparison, and the trend test developed by Cuzick for testing trend [19]. The level of significance is set at 0.05 (two-sided). Data are presented as mean ± one standard error of the mean [19]. Aggregates of fluorescence cells were seen in sections with relatively high levels of marrow-to-muscle conversion. We have defined muscle colonies as three or more adjacent fibers.

Results

Bone marrow cells can participate in regeneration of the muscle fibers after injury

The myogenic potential of bone marrow cells was studied by tracking the transplanted cells from GFP-transgenic mice (Jackson Laboratories, Bar Harbor, ME, USA). In these mice 100% of skeletal muscle cells were positive for GFP. C57BL/6 animals were transplanted with 25 million bone marrow cells from GFP^+ mice after 900 cGy of whole-body radiation. Two months after transplantation high peripheral blood donor chimerism was determined by FACS analysis for GFP^+ cells (60–75%). Right tibialis anterior muscle was then injected with cardiotoxin and the left tibialis anterior muscle received no injury. In a repeat experiment left tibialis anterior muscle was injected with PBS. One and two months after injury animals were sacrificed and their tibialis anterior muscle was resected and fixed. GFP^+ cells were identified by their green fluorescence under fluorescent microscopy (Fig. 1C and E). Presence of GFP was further confirmed by using an anti-GFP antibody (Fig. 1C and D). Chimeric muscle fibers were characterized by distinct morphology, size, lack of positivity for CD45, and positive staining for desmin and dystrophin (Fig. 1G–L). Inappropriate fixation methods could result in bleeding of GFP in the neighborhood cells. In such cases a green background can be seen not only in the cells but in the surrounding glass slide. Those slides were removed from analysis and only GFP^+ cells with clear and discrete borders were counted. With the help of special software, three-dimensional reconstructions of the muscle fibers were made to show that GFP^+ hematopoietic cells were not overlapping on skeletal myocytes (Fig. 2).

Effect of radiation

We further investigated the effect of the radiation on marrow-to-muscle conversion. Five C57BL/6 mice received 20 million bone marrow cells from GFP^+ mice followed by

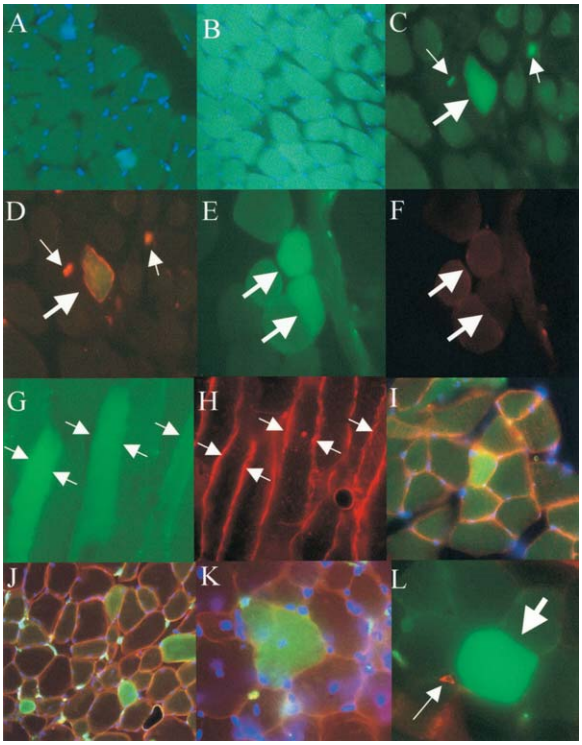


Figure 1. Digital pictures of frozen sections of skeletal muscle from **(A)** normal C57BL/6 mouse and **(B)** a GFP-transgenic animal. Notice that 100% of the muscle fibers are positive for green fluorescent protein in the transgenic. **Panels C–L**: GFP^+ muscle fibers were evaluated in tibialis anterior muscle sections of animals transplanted with 25 million GFP^+ marrow cells after 900 cGy of irradiation. Two months later tibialis anterior muscle was injected with cardiotoxin followed by intravenous injection of 20×10^6 GFP^+ marrow cells the next day and they were evaluated one month after injury **(C)** GFP fluorescense is seen with FITC filter (arrows), confirmed in panel **D** with GFP antibody conjugated to anti-rabbit-Texas Red. Larger arrow show a GFP^+ muscle fiber and small arrows demonstrate hematopoietic or other cells. **(E)** Arrows show two GFP^+ fibers. **(F)**: Staining with an isotope control antibody for anti-GFP antibody conjugated to Texas Red demonstrates that there is no background for the two GFP^+ fibers shown in panel E. **(G)**: Multiple GFP^+ fibers (arrows) show desmin signal (desmin conjugated to Alexa Fluor 594) in **H**. **(I)**: Digital overlapping of pictures taken from a muscle section stained with desmin demonstrates a GFP^+ fiber stained with desmin (red). **(J, K)**: Digital overlapping of pictures taken from sections that were double-stained for GFP antibody (green) and dystrophin (red). **(L)**: double-staining with anti-GFP (green) and anti-CD45 antibodies demonstrate that all muscle fibers including GFP^+ fibers (thick arrow) were negative for CD45, while the smaller cell between the muscle fiber (thin arrow) is positive for this antigen (i.e., they are hematopoietic in origin). DAPI nuclear staining (blue) can be seen in panels A, B, I, J, and K. Magnification 20× for panels A, B, and G–J and 40× for panels C–F and K–L.

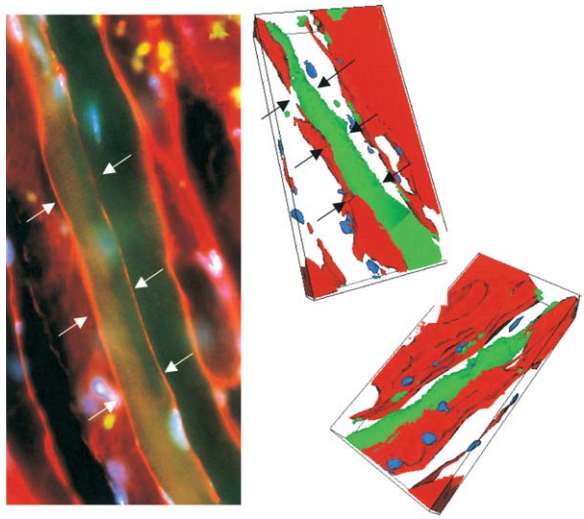


Figure 2. Three-dimensional reconstruction of a GFP^+ muscle fiber in C57BL/6 mouse. Animal was transplanted with 20 million marrow cells from GFP-transgenic mouse followed by injury to the right tibialis anterior 3 weeks after transplant and intravenous injection of 20 million GFP marrow cells into the mouse the next day after injury. Muscle samples were evaluated at 4 weeks after injury. Dystropin is visualized by Alexa Fluor 594 (red) and cell nuclei by Dapi staining (blue). 3D reconstruction of the section on the left shows that there is no overlapping of other GFP cells over the muscle fiber (shown between the arrows) and GFP (green) is within the muscle fibers. It also shows that the GFP^+ muscle fiber is engulfed by dystrophin staining. Original magnification was 40×.

cardiotoxin injury to the tibialis anterior muscle, one month after transplantation. One month after injury, animals were sacrificed and frozen sections were analyzed for the presence of GFP^+ fibers. Two of the animals showed no GFP^+ fibers and the level of chimerism in the other three was very low (the average conversion rate for the whole group was 0.066% ± 0.037%, n = 5). Increasing the number of infused marrow cells to 100 million cells also did not change the outcome (0.046% ± 0.020%, n = 4). However, when 20 million GFP marrow cells were infused after 500 cGy of whole-body and 500 cGy of lower-extremity radiation and animals were injected similarly with cardiotoxin, almost 20-fold more GFP^+ fibers were identified (1.290% ± 0.299%, n = 5).

Importance of injury

Marrow from GFP-chimeric animals (900 cGy of radiation and 40 million marrow cells) were mobilized with different mobilization regimens around the time of cardiotoxin injury to the right tibialis anterior muscle as was described in the methods section. Muscle specimens were analyzed one month later for the presence of GFP^+ muscle fibers. In all the animals, regardless of the type of mobilization, much lower levels of chimerism were seen on the left muscles (mean of 0.01% ± 0.18%) vs the right muscle (mean of 1.13% ± 0.14%) with a *p*-value of 0.0003 (n = 9 animals in each group). In a similar experiment the percentage of marrow-derived muscle fibers was compared between the muscle fibers that received cardiotoxin and those that received no injury or were injected with PBS alone. Interestingly, even PBS injection alone increased the number of

GFP$^+$ cells (Fig. 3). We have evaluated a total of 58 additional mice for GFP$^+$ muscle fibers, comparing the noninjured (left) to injured (right) anterior tibialis muscles. In all cases, without exception, there was striking difference between the groups with very low level of donor-derived fibers in the noninjured and significant increase in fibers in the injured sides.

In multiple different experiments with different protocols that included more than 50 mice, we universally found significant increases in the number of the GFP$^+$ muscle fibers in the right tibialis anterior muscle that received cardiotoxin in comparison with the left tibialis anterior muscle that was not injured (data not shown).

Type and schedule of bone marrow mobilization affects the level of marrow-derived muscle chimerism

To evaluate the effects of different mobilizations, we transplanted C57BL/6 animals with 40 million bone marrow cells from GFP-transgenic mouse after 900 cGy of radiation. Two months later, after confirmation of chimerism by peripheral blood analysis, we injected 100 μL (10 mM in 0.9% NaCl) of cardiotoxin (Sigma, St. Louis, MO, USA) into the anterior tibialis muscle. Starting at day -3 for a total of 5 days, bone marrow stem cells of the animals were mobilized by subcutaneous injection of different cytokines. The first group received no mobilization; second and third group received 1 and 2 cycles of G-CSF mobilization (250 mg/kg/day), respectively; fourth group had G-CSF (50 mg/kg/day) and steel factor (200 mg/kg/day); and the fifth group was injected with GM-CSF (1 mg/mouse/day). One month later, the right and left anterior tibial muscles were isolated. In the group that received no mobilization, injured muscle showed some GFP$^+$ myocytes. In groups that received G-CSF alone or GM-CSF, the number of positive myocytes increased. The highest numbers of GFP$^+$ myocytes were seen in the group that had G-CSF plus steel factor ($p < 0.034$) and the group that had 2 cycles of mobilization with G-CSF 1 week apart ($p < 0.021$) (Fig. 4).

Direct injection of bone marrow cells increases the number of donor-derived muscle fibers

Ten million bone marrow cells from GFP-transgenic mice were transplanted to C57BL/6 mice after 500 cGy of total-body radiation and 500 cGy of lower-extremity radiation. Two weeks later, the recipients received intramuscular injections of cardiotoxin followed by direct injections of PBS or different populations of bone marrow cells the next day. Noninjured tibialis anterior muscle on the left side was used as a control. Samples were analyzed one month after injury. In all experimental groups, the number of GFP$^+$ cells on the left side remained less than 0.01%. Injection of 4 million bone marrow cells into muscle resulted in a small increase in the number of the GFP$^+$ myocytes when compared with injection of PBS alone (data not shown). Injection

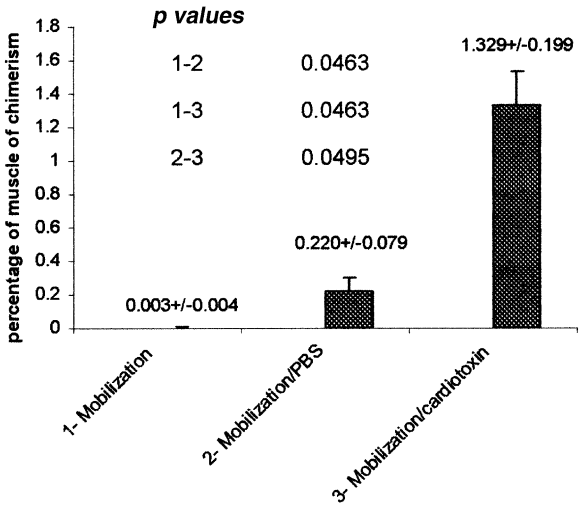


Figure 3. Diagram demonstrates the percentage of GFP$^+$ muscle fibers in animals without any intervention (1) and after injection of 100 μL of PBS (2) or cardiotoxin (3). All animals received bone marrow mobilization with G-CSF 2 months after transplantation of 40 million GFP$^+$ marrow cells from GFP-transgenic animals. Cardiotoxin was injected directly into the tibialis muscle at the time of mobilization and after confirmation of donor-derived chimerism (see the result section for details of transplantation). Animals were analyzed one month after cardiotoxin injury. Note that while cardiotoxin results in diffuse severe injury of the muscle fibers, injection of PBS causes relatively mild injury which enhances the conversion of marrow cells to muscle fibers n = 3.

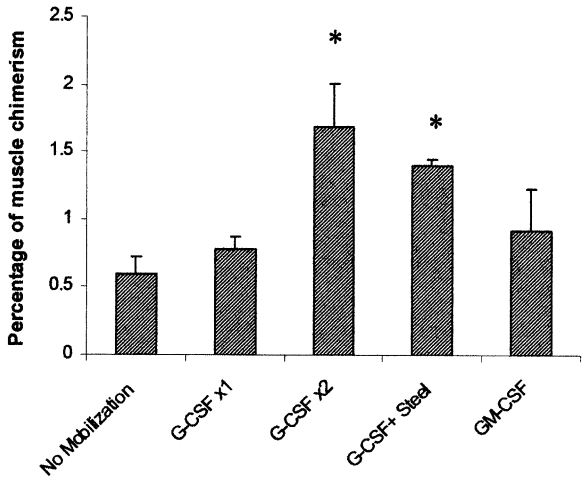


Figure 4. Comparison of different modes of mobilization on the quantity of marrow-derived muscle chimerism. Animals were first transplanted with 25 million bone marrow cells from GFP-transgenic mouse after 900 cGy of radiation. Two months later, after establishment of stable chimerism, they underwent muscle injury with cardiotoxin at day 0. Starting day −3 before injury daily, for total of 5 days, they received G-CSF alone or with steel factor or GM-CSF. One group received two cycles of G-CSF two weeks apart. Mice were sacrificed one month after injury and tibilas anterior muscle were analyzed for GFP$^+$ muscle fibers. Analysis of a second group 2 months after injury showed very similar data. Each bar represents 3 to 4 animals from two separate experiments. *The difference between no mobilization and G-CSF×2 or G-CSF plus steel factor are significant (p-value of 0.034 for each). Also the difference between one cycle of G-CSF and 2 cycles of G-CSF plus steel are significant (p-value of 0.021).

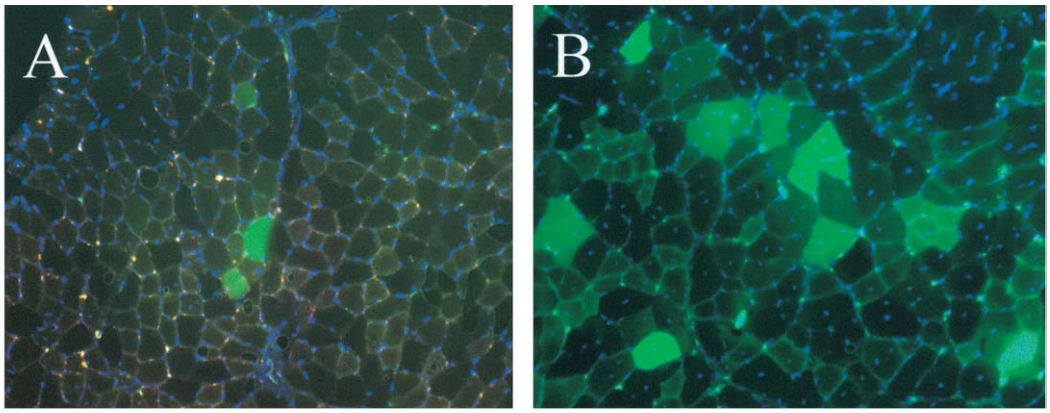
of 6×10^5 lineage-depleted marrow cells resulted in much higher marrow-derived muscle chimerism than whole marrow ($p < 0.01$). Staining with anti-GFP antibody that enhances the weaker signals of GFP in these specimens demonstrated that up to 12% of the skeletal myocytes in some of the sections were positive for GFP (Fig. 5B, animal 5) while the incidence of positivity in control muscle (left leg of the same animals) was still less than 0.01%.

Importance of the marrow tracking methods

To compare our results with some other reports of marrow-to-muscle conversion [13,16], we used a β-galactosidase-expressing Rosa mouse, which is commonly used for tracking of donor marrow cell after their transplantation. X-gal staining of muscle section in a Rosa mouse showed that 100% of the fibers were positive for X-gal. Similar to our experiments with GFP mice, C57BL/6 mice were transplanted with 20×10^6 bone marrow cells from ROSA26 mice after 500 cGy of total-body and 500 cGy of lower-extremity radiation. One month after radiation, cardiotoxin was injected directly into tibialis anterior muscle followed by intramuscular injection of 4×10^6 bone marrow cells from ROSA26 mice the next day. One month later animals were sacrificed. Evaluations of frozen sections of muscle stained with X-gal showed that the majority of hematopoietic cells in the muscle vasculature were donor derived. However, only very rare β-galactosidase$^+$ skeletal muscle cells were identified (two positive cells in greater than 2000 fibers counted). Thus in our hands, conversion rates were much lower using a β-galactosidase marker system as compared to a GFP marker system.

GFP$^+$ muscle cells can form muscle colonies

Clusters of GFP$^+$ muscle fibers were found in many of our regenerative muscle experiments (Fig. 6). The phenomenon not only occurred in the samples that received direct injection of marrow cells but also was seen in the experiments in which marrow cells were mobilized after muscle injury. In six samples from two different animals who received GFP$^+$ lineage-negative cells intramuscularly, we counted a



A	fibers counted	GFP+ fibers	Ratio
animal 1	6659	96	**1.44%**
animal 2	5925	106	**1.79%**
animal 3	3504	54	**1.54%**
animal 4	6463	120	**1.86%**
animal 5	8347	153	**1.83%**
animal 6	5936	73	**1.23%**

B	fibers counted	GFP+ fibers	Ratio
animal 1	3424	220	**6.42%**
animal 2	4859	168	**3.46%**
animal 3	3943	88	**2.23%**
animal 4	4915	208	**4.23%**
animal 5	1917	235	**12.26%**
animal 6	3006	245	**8.15%**

Figure 5. Direct injection of lineage-negative bone marrow cells. Tibialis anterior muscle of C57BL/6 mice that were transplanted with 10×10^6 GFP$^+$ marrow cells after 500 cGy of total-body radiation and 500 cGy of lower-extremity radiation were injured with cardiotoxin. One day after injury GFP$^+$ whole marrow population **(A)** or its lineage-negative population **(B)** were directly injected to the injured muscle. Analysis of muscle fibers 4 weeks after injury are summarized above. Figures represent digitally overlapped photomicrographs of frozen sections of the muscle after GFP antibody (green) and DAPI (blue) staining taken with FITC and DAPI filters. Results demonstrate combined data from two similar experiments. The average percentage of GFP$^+$ muscle fibers was 1.62% ± 0.10% for the group that received whole bone marrow, which is significantly less than the group that received lineage-negative population (6.13% ± 1.50% with $p < 0.01$). In the latter group some sections showed as high as 12% marrow-derived muscle chimerism. There is obvious heterogeneity for GFP fluorescence between individual GFP$^+$ fibers. Magnification 20×.

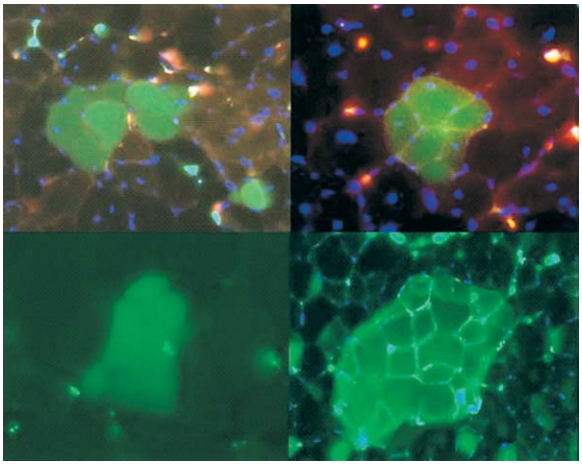


Figure 6. GFP$^+$ muscle colonies can be seen in regenerative muscle after cardiotoxin injury. B6 mice were transplanted with 20 million GFP$^+$ bone marrow cells after 900 cGy of radiation and one month later cardiotoxin was injected into tibialis anterior muscle. From three days before injection for the total of 5 days animals received G-CSF for mobilization of bone marrow cells. Evaluation was carried out at 4 weeks after injury. Some of the colonies had 20 or more GFP$^+$ muscle fibers. Comparing with individual fibers in Figure 5, there is striking homogeneity for GFP fluorescence within the same colonies. Magnification is 40× for all panels.

total of 643 GFP$^+$ muscle fibers, including 44 muscle colonies. We further graded the intensity of the green fluorescence in the single cells to dull, moderate, or bright. Interestingly in 95% ± 0.85% of the colonies counted, the intensity of GFP expression was identical in fibers within the same colony, while for single GFP$^+$ fibers dispersed throughout the specimen the probability of three cells (the minimum needed for a colony based on our definition) being the same color was only 14.12%. Based on these observations the probability of a minimum of three fibers in the colony to be homogenous by random association is less than 0.0001. We also compared the number of the colonies in two different experimental settings. In both settings, GFP$^+$ marrow cells were injected intravenously after 900 cGy of radiation to achieve marrow chimerism and one month later both groups received cardiotoxin injury to the tibialis anterior muscle. In one protocol animals received two cycles of G-CSF for mobilization as described earlier and in the other experiment they received lineage-negative GFP$^+$ marrow cells directly into their tibialis anterior the day after injury. Analysis of multiple sections in two animals from each group demonstrated that in individual sections the ratios between GFP$^+$ colonies and the total number of GFP cells were very similar (6.79% ± 0.69% for the direct injection protocol and 5.88% ± 0.86% for mobilization group).

Fusion of marrow cells to regenerating muscle cells

We studied the possibility of fusion of marrow cells to muscle fibers in three different models of transplantation. In each model, 15 to 20 million bone marrow cells from the donor were transplanted to the recipient after 500 cGy of whole-body and 500 cGy of local radiation to the leg. One month later, cardiotoxin was injected to the right tibialis anterior muscle and the next day the same muscle received 2 million donor cells by direct injection and the muscle samples were analyzed one month after injury. In the first model, transplantation of male GFP$^+$ marrow into C57BL/6J female host mice resulted in rare Y-chromosome signal in GFP$^+$ muscle fibers regenerating after cardiotoxin injury, but the same muscle fibers also had many other nuclei without a Y-chromosome signal (Fig. 7A and B). In the second model of female GFP to male C57BL/6 transplant, we demonstrated that all of the GFP$^+$ fibers had Y-chromosome-positive nuclei (Fig. 7C and D). In the third model, bone marrow cells from male GFP-transgenic mice were transplanted into male β-galactosidase (ROSA26) mice and animals underwent cardiotoxin injury to the tibialis anterior muscle. Costaining with anti-GFP antibody and X-gal for β-galactosidase demonstrates that many of the GFP$^+$ fibers were simultaneously stained with X-gal (Fig. 7E–H).

Discussion

The present data indicates that a converting cell in marrow can form a significant number of skeletal muscle fibers mediated by fusional events. Furthermore, radiation and muscle injury is a critical factor in such conversions and stem cell mobilization can increase the number of such conversions, as can a direct injection of marrow cells into muscle or purification of marrow progenitor/stem cells.

We use the term "conversions" with due forethought. Controversy has swirled unnecessarily around the idea that marrow stem cells "transdifferentiate" into nonmarrow cells. Transdifferentiation has yet to be absolutely proven in any system. We hypothesize that marrow cells with wide differentiation potential express that potential when placed in the appropriate environment with strong inductive signals. Our data further indicate that cell fusion is part of this process. For skeletal muscle this was not surprising, since fusion is a part of normal muscle regeneration, and after injury activated satellite cells can generate myoblasts that in turn fuse to the existing muscle fibers. Fusion, of course, is simply one of several potential mechanisms to an end. The elegant work by Wang et al. [20] in the FAH–mouse, with genetic liver failure, has shown that fusion-mediated events can produce a large number of progeny cells with the ability to reverse a disease process. Since most muscle cells are formed by fusion, it is not surprising that fusion is involved in the conversion events. Whether marrow initially converts to muscle or directly fuses is for further studies, although work by LaBarge et al. [17] suggests a sequence of conversion to satellite cells with subsequent fusion. It is important to note that fusion appears to be model and injury dependent and it is not seen in other structured experimental settings.

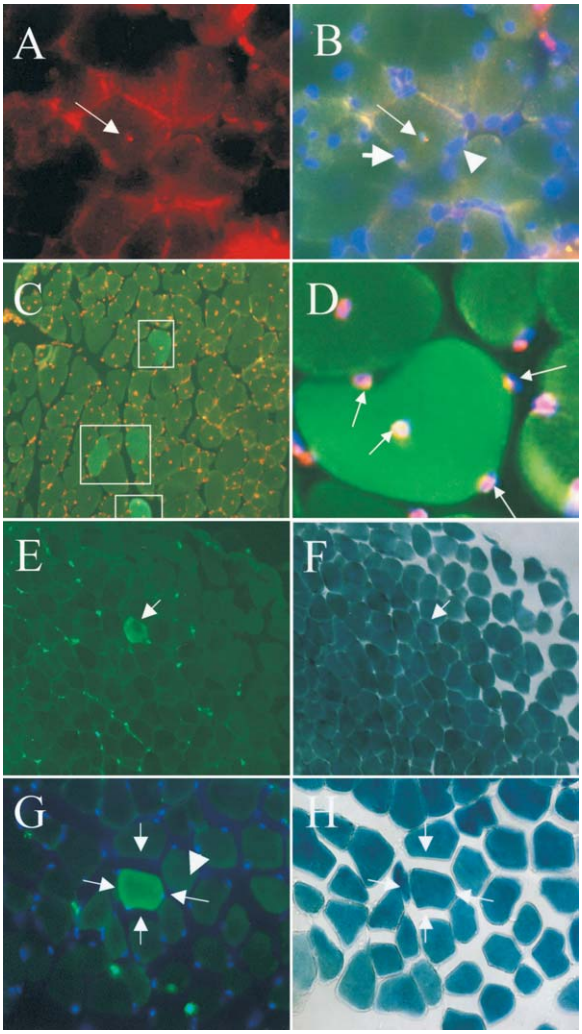


Figure 7. Marrow cells fuse to regenerating muscle. **A–B:** Photomicrograph of FISH staining on muscle frozen sections of a female C57BL/6 mouse transplanted with marrow cells from a male C57BL/6 mouse. Long arrows demonstrate signal for Y-chromosome. In A, picture is taken with a rhodamine filter and in B, picture is a digital overlap of rhodamine, FITC, and Dapi (nuclear staining). Short arrows in B demonstrate other nuclei within the membrane of the same muscle cells without the Y-chromosome signal. **Panels C and D** show FISH staining in frozen sections of the tibialis anterior muscle of male C57BL6 mice transplanted with female GFP marrow cells followed by cardiotoxin injury. In C, boxes show GFP^+ muscle fibers with Y-chromosome signals (red dots) in their nuclei. **Panel D** demonstrates higher magnification of another GFP^+ muscle fiber with arrows pointing to Y-chromosome signal. **E–H:** Muscle from ROSA26 mice transplanted with GFP marrow cells after cardiotoxin injury. E shows GFP immunofluorescent staining of a GFP^+ fiber corresponding to a X-gal-positive cell in F (arrow). G and H show higher magnification of the same phenomenon. Magnification is 20× for C, E, F, G, and D; 40× for A and B, and 60× for C.

Our studies indicate that injury is a critical component. Cardiotoxin injury resulted in relatively high levels of GFP conversions while the uninjured control mice show only very rare events. Significantly, even the minor injury of a PBS injection showed an increased number of conversions. One theme of positive plasticity studies has been injury and selection. In the work of Lagasse et al. [7], the selection was continuous over time by repetitive withdrawal of 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3 cyclohexanedione TNBC, which corrects the manifestation of the genetic defect. Other injuries such as cardiotoxin would be massive and long lasting. In our studies, there were actually two injuries, the original irradiation and cardiotoxin. We found radiation as a critical factor in marrow-to-muscle conversion since without radiation the level of GFP^+ fibers was negligible. The mechanism of such an effect is not clear at this point; however, we speculate that it may work by establishing marrow chimerism and/or by depleting muscle stem cells. Data from studies on liver conversions support these two injury hypotheses. One injury may predominantly decrease competing tissue stem cells. The other, a more severe local damage, may generate homing and selection peptides. We believe that some of the controversy and negative reports in the stem cell plasticity field appear to be due to the lack of appropriate injury.

Stem cell mobilization is a common practice to obtain stem cells for conventional therapeutic stem cell transplants. In our studies we used G-CSF, two cycles of G-CSF, G-CSF plus steel, or GM-CSF to mobilize endogenous marrow stem cells and observed changes in the extent of GFP^+ marrow cell conversion to anterior tibialis muscle cells. More was clearly better, as two cycles of G-CSF were superior to other mobilizations. This suggests that increasing the number of mobilizations over time might continue to increase the extent of conversions. The type of mobilized cells and their fate in the mouse is unclear. Besides the stem cells, other cells like monocytes and macrophages and perhaps endothelial precursor cells may be mobilized by the same cytokines. Some may return to marrow, some may be trapped in the "wrong" tissue and die; but others, as suggested by these data, may home to an injured tissue and convert or fuse to cells of that tissue. Furthermore, these cytokines may have a direct local effect on marrow-to-muscle conversion.

These studies showed the presence of GFP^+ muscle colonies. The muscle colonies were seen both after intravenous or direct injection and have one striking feature; the cells in the colonies were homogeneous with regard to degree of GFP fluorescence, while individual isolated fibers were quite heterogeneous. Heterogeneity of the GFP fluorescence probably reflects the number of GFP fusional events per existing muscle fiber. It is difficult to explain the homogeneity of the fibers in a colony by current understanding of muscle regeneration that suggests satellite cells repair muscle damage by fusion into the existing muscle fibers. One possibility is that a GFP^+ satellite cell can divide into multiple cells with similar fluorescence that eventually fuse in a rather similar ratio to themselves or to the surrounding fibers. On the other hand, marrow-derived cells may be transformed to more primitive myogenic cells that behave differently from satellite cells and directly produce fibers. These of course remain to be proved.

The present studies show levels of muscle fiber replacements of up to 12%. This, to our best knowledge, demonstrates the highest number of bone marrow–derived myocyte regeneration reported in the literature. These levels begin to approach levels that might be useful in the treatment of muscle disorders, particularly muscular dystrophy. The work by Lagasse et al. [7] indicates that hepatic cells replaced may be adequate to ameliorate a disease process. We anticipate that by combining the appropriate injuries, cell type, and mobilization we will be able to further increase the degree of marrow-to-muscle conversions until it becomes therapeutically useful.

Finally, it is important to indicate that these conversions are real, as indicated by characteristic muscle morphology observed both by conventional microscopy and three-dimensional reconstruction of the GFP^+ muscles, lineage-specific marker colocalization with donor marker (GFP), and the lack of the hematopoietic marker CD45.

The present study has sought to elucidate treatments that would maximize conversion events, not to identify the specific marrow cell types involved, although others have shown that highly purified hematopoietic marrow stem cells can convert to a variety of tissue cells [7,9]. We have shown that the converting cell is enriched in lineage-negative cells, and a future goal is to further subset marrow and try to identify the cell population mediating the conversions. In any case, the best speculation at present is that these conversion events are mediated either by a mesenchymal stem cell, a hematopoietic stem cell, or a mixture of the two. However, other defined and undefined cell types could be responsible.

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